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Abstract

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PPARβ/δ affects pancreatic β cell mass and insulin secretion in mice

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PPARβ/δ protects against obesity by reducing dyslipidemia and insulin resistance via effects in muscle, adipose tissue, and liver. However, its function in pancreas remains ill defined. To gain insight into its hypothesized role in β cell function, we specifically deleted Pparb/d in the epithelial compartment of the mouse pancreas. Mutant animals presented increased numbers of islets and, more importantly, enhanced insulin secretion, causing hyperinsulinemia. Gene expression profiling of pancreatic β cells indicated a broad repressive function of PPARβ/δ affecting the vesicular and granular compartment as well as the actin cytoskeleton. Analyses of insulin release from isolated PPARβ/δ-deficient islets revealed an accelerated second phase of glucose-stimulated insulin secretion. These effects in PPARβ/δ-deficient islets correlated with increased filamentous actin (F-actin) disassembly and an elevation in protein kinase D activity that altered Golgi organization. Taken together, these results provide evidence for a repressive role for PPARβ/δ in β cell mass and insulin exocytosis, and shed a new light on PPARβ/δ metabolic action.

Introduction

High plasma glucose levels due to insulin resistance and inadequate insulin production characterize type 2 diabetes. Generally considered an adult illness, it is now often diagnosed in teenagers in parallel with rising obesity rates. Type 2 diabetes is a multigenic disease, and several genome-wide association studies have identified many novel loci, most seeming to influence β cell capacity to enhance insulin secretion in response to increased insulin resistance or body weight (1, 2). Many of the identified insulin secretory defects remain poorly understood at the molecular level, especially the decline in β cell mass and function.

Mechanisms regulating adult β cell mass include proliferation of preexisting β cells (3) and β cell neogenesis from ductal precursor cells (4). Tight regulation of β cell mass is required for preserving insulin secretion capacity over a lifetime. In fact, glucose-induced pancreatic β cell expansion supports the notion of existing β cell replication in the adult organism, but the mechanisms of β cell homeostasis and regenerative repair are not well understood.

In rodents, glucose-stimulated insulin secretion (GSIS) exhibits a biphasic pattern characterized by a rapid (5–10 minutes) first-phase release, followed by a second phase of slow, pulsatile release (5, 6). This pattern also occurs in humans (7) and is important for insulin to fulfill its regulatory function (8). The increase of cytoplasmic Ca2+ triggered by enhanced circulating glucose levels induces a first-phase release that corresponds to exocytosis of a readily releasable pool (RRP) of insulin-containing granules predocked beneath the cell surface and granules newly recruited that fuse immediately to the plasma membrane (9). A more enduring second-phase release follows, in which new granules are recruited from a reserve pool (RP) by processes referred to as priming (10). Among the molecular mechanisms regulating RRP and RP, the diacylglycerol-activated (DAG-activated) protein Munc13-1 (11) and the GTP-regulated protein Rab3 (12) are involved in granule priming in pancreatic β cells. Furthermore, capacitance measurements and perfusion experiments using cell lines and mouse islets have demonstrated that cytoskeleton regulator proteins also affect priming (13–16). Finally, the p38α/PKD1 pathway integrates regulation of the insulin secretory capacity and β cell survival (17).

None of the above-mentioned studies have, however, revealed a master regulator that would coordinate glucose sensing and insulin secretion from granule formation to exocytosis. Free fatty acids have complex regulatory roles in insulin secretion (18). Acute treatment of islets with palmitate potentiates GSIS in the β cell, both via intracellular metabolic lipid signaling (19) and the free fatty acid receptor GPR40 (20). Another fatty acid receptor that plays a role in insulin secretion is PPARα (NR1C1). In fact, we showed that pancreatic islet adaptation to fasting is dependent on PPARα transcriptional upregulation of fatty acid oxidation (21). A second PPAR that has been shown to play a role in β cell function is PPARγ (NR1C3) (22).

Little is known about PPARβ/δ (NR1C2), the third member of the family. Glucose-induced lipid peroxidation of arachidonic acid and linoleic acid generates endogenous ligand of PPARβ/δ, which induces insulin secretion in INS-1E and rat isolated islets (23). Hence, we hypothesized that the fatty acid receptor PPARβ/δ may play a general regulatory role in these cells. This receptor shows a broad expression pattern and controls processes such as the inflammatory response, cell differentiation, and survival (24–30). So far, PPARβ/δ has been implicated in systemic glucose and lipid homeostasis by its action in nonpancreatic tissues. The PPARβ/δ ligand GW501516 reverses meta-
bolic abnormalities associated with obesity, such as dyslipidemia and insulin resistance, in humans, monkeys, and mice (31–33).

The benefits of PPARβ/δ have been attributed to its induction of genes related to fatty acid oxidation and energy dissipation in adipose tissue and muscle (34, 35). Furthermore, it has been proposed that PPARβ/δ protects GSIS against adverse effects associated with prolonged fatty acid exposure in INS-1E and HIT cells (36, 37). However, its role in vivo remains to be explored. Here, we report the generation and analysis of pancreas-specific Pparb/d-null mice, which revealed an unexpectedly broad repressive role for PPARβ/δ on pancreatic β cell proliferation and function.

**Results**

**Pancreatic PPARβ/δ expression.** PPARβ/δ expression in mouse pancreas is poorly characterized. However, it is known to occur in rat pancreas from E15.5 in both the endocrine and exocrine compartments (24). We found increased Pparb/d mRNA expression during mouse pancreas development (Figure 1A), with levels 3 times higher at E17.5 than at E13.5 and E15.5. At P14, Pparb/d mRNA levels had increased 2-fold versus those at P2, but then decreased at 2 months of age after exocrine pancreas expansion. The RNA expression pattern of PPARβ/δ is more similar to that of PPARα than of PPARγ (Supplemental Figure 1, A and B; supplemental material available).
online with this article; doi:10.1172/JCI42127DS1). FACS-sorted cells from adult mouse islets showed that PPARβ/δ was expressed in both α and β cells (Figure 1B). PPARβ/δ is also expressed in human islets, although slightly less than PPARα (Figure 1C).

Considering the important expression of PPARβ/δ in both mouse and human islets and the whole mouse pancreas (Figure 1C and Supplemental Figure 1C), we concentrated on elucidating its functions in pancreatic β cells.

Deletion of Pparb/d in the pancreas. Deletion of Pparb/d or over-activation of PPARβ/δ, specifically in muscle and adipose tissue, affects insulin resistance and obesity (34, 35). To avoid indirect effects of whole-organism inactivation of Pparb/d on pancreas functions, we generated mice lacking PPARβ/δ specifically in the pancreas. These animals were generated by breeding mice harboring a floxed Pparb/d allele were verified by PCR amplification from pancreatic genomic DNA (Figure 1E). The detection of an amplified 490-bp DNA fragment in Pdx1Cre;Pparb/dfl/fl (lanes 3 and 4) and Pdx1Cre;Pparb/dfl/+ (lanes 5 and 6) mice demonstrated the excision of the floxed Pparb/d allele (fl). The 2-kb DNA fragment in the absence of the Cre transgene (lanes 1 and 2) or presence of the WT Pparb/d allele (+) (lane 5–7) demonstrated that the deletion was indeed only present in Pdx1Cre;Pparb/dfl/+ and Pdx1Cre;Pparb/dfl/fl mice, confirmed by the absence of Pparb/d mRNA in pancreas and islets isolated from 8-week-old Pdx1Cre;Pparb/dfl/fl mice (Figure 1F and Supplemental Figure 1D).

Elimination of PPARβ/δ did not trigger upregulation of PPARγ and PPARα (Supplemental Figure 1, F and G).

PPARβ/δ deficiency induces hyperinsulinemia and decreased glycemia starting at 4 weeks of age. The Pdx1Cre;Pparb/dfl/fl mice were born at the expected Mendelian ratio and showed normal growth and develop-
Precocious inactivation of Pparb/d in the pancreas leads to increased islet numbers. The next question was whether Pparb/d excision affects β cell development, which would explain the observed alterations of circulating insulin and glucose levels. Because the Pdx1 promoter, which controls Cre recombinase expression, becomes active in the foregut epithelium just before the onset of pancreatic bud formation, we assessed alterations in β cell differentiation in the embryonic pancreas. We quantified endocrine precursor neurogenin 3–expressing (Neurog3-expressing) cells and insulin-expressing cells by immunohistochemistry in the E15.5 pancreas. We detected no significant difference between Pdx1Cre;Pparb/dβ−/δ− and Pparb/dβ−/δ− embryonic pancreata and no difference in Insulin I and Neurog3 mRNA levels (Supplemental Figure 2, A–D). Furthermore, there was no alteration in protein level of Neurog3 in pancreas from Pdx1Cre;Pparb/dβ−/δ− compared with Pparb/dβ−/δ− embryos at E17.5 and E18.5 (Supplemental Figure 2E). However, quantitative morphometry demonstrated a 2-fold increase in β cell mass and a 1.5-increase of β cell mass in 8-week-old Pdx1Cre;Pparb/dβ−/δ− mice (Figure 3A and Supplemental Figure 2F) due to increased islet number (Figure 3B and Supplemental Figure 2F). The increase in BrdU incorporation in β cells at 2 weeks of age (Figure 3C) suggested that their higher proliferation at this stage may contribute to the increased islet mass in 8-week-old Pdx1Cre;Pparb/dβ−/δ− mice. At later stages, this difference in islet cell proliferation between PPARβ/δ-deficient and WT animals was not recorded anymore.

To characterize the impact of this β cell mass expansion, we measured pancreas insulin content at P3 and in 2-, 4-, and 8-week-old mice. At P3, PPARβ/δ mutants presented no alteration in total pancre
creatic insulin content (Figure 3D). However, there was a trend toward an increased insulin content in 2-week-old Pdx1Cre;Pparb/dfl/fl mice, which became significant at 4 and 8 weeks with a 2-fold increase compared with controls (Figure 3D). There was no difference in insulin mRNA or protein content per β cell in islets isolated from 8-week-old Pdx1Cre;Pparb/dfl/fl mice and Pparb/dfl/fl control littermates (Figure 3, E and F). Thus, the increase in islet numbers may account for the augmented pancreatic insulin content. Even though in our model, α cells also present the Pparb/d deletion, no significant alteration of glucagon content, neither in islets (Supplemental Figure 2G) nor pancreas (Supplemental Figure 2H), was recorded. Collectively, these data showed that Pparb/d deletion in pancreas enhanced β cell mass by increasing the number of islets and that the enhanced proliferation of islet cells occurred during the early postnatal period.

Pparb/d deletion in islets enhances second-phase insulin secretion. Because increased β cell number does not always account for hyperinsulinemia and hypoglycemia (39, 40), we tested whether other factors are involved in the phenotype we observed. Thus, we looked into altered β cell functions in Pdx1Cre;Pparb/dfl/fl islets, particularly in relation to insulin secretion.

First, we measured GSIS in perifused islets isolated from 8-week-old mice (Figure 4, A and B). At basal levels of glucose (2.8 mM), insulin secretion did not measurably differ between mutated and control islets. In elevated glucose (16.7 mM), islets from Pdx1Cre;Pparb/dfl/fl mice exhibited higher insulin release than control islets during second-phase secretion, starting approximately 10 minutes after glucose stimulation. In contrast, insulin secretion during first-phase (first 10 minutes of 16.7 mM glucose induction) was similar in Pdx1Cre;Pparb/dfl/fl and control islets (Figure 4A).

Second, we characterized the enhanced insulin secretion of Pdx1Cre;Pparb/dfl/fl islets by monitoring exocytosis with whole cell membrane capacitance measurements. During exocytosis, the granular vesicle membrane is incorporated into the plasma membrane, increasing the cell surface. Membrane capacitance is proportional to the cell surface area and is routinely used to measure exocytosis with high temporal resolution in single cells. A single insulin granule will typically contribute to a capacitance increase of approximately 3.5 fF. We noted that initially the mutated cells were larger than controls (8.8 ± 0.4 pF vs. 6.1 ± 0.4 pF, respectively; \( P < 10^{-4} \)). Exocytosis was elicited by a train (1 s−1) of 10 voltage-clamp depolarizations from −70 to 0 mV, each lasting 500 ms (Figure 4C). The first depolarization triggered a capacitance increase of 153 ± 57 fF in control islets, not different from the 161 ± 57 fF measured in mutated islets (Figure 4D). How-
Figure 5
Gene expression profiling in PPARβ/δ-null islets. (A) Average expression (log2) across the 3 Pparb/dfl/fl control mice and the 3 Pdx1Cre;Pparb/dfl/fl KO mice versus log2 fold-change between KO and control. Probe sets with a higher expression in KO have a positive fold change. Red dashed lines indicate 1.5-fold and 2-fold changes. Probe sets significantly different at an FDR of less than 5% are indicated in blue and an FDR of more than 5% and less than 10% in green. (B) Enrichment of GO annotation terms. Enrichment for annotation terms was performed separately on upregulated and downregulated genes with an FDR of less than 5% or an FDR of less than 10%. Shown are observed (Obs) and expected (Exp) numbers of regulated genes for significant terms. *P < 0.05; **P < 0.01. (C) Relative mRNA expression levels in islets from 8-week-old mice determined by qPCR (n = 3). *P < 0.05; **P < 0.01. (D) Effect of 100 nM of GW501516 (GW) incubation during 24 hours on GSIS from control and KO islets (n = 3). *P < 0.05.
ever, subsequent depolarizations evoked much larger increases in the mutated cells. The summed response during depolarizations 2 to 10 increased capacitance by 170 ± 77 fF in controls, less than a third of the 551 ± 144 fF in mutated cells (Figure 4D, *n* = 15 vs. 11, *P* < 10⁻⁴).

The increase in evoked exocytosis is unlikely to be due to accelerated influx through voltage-gated Ca²⁺ channels because the charge and peaks of the associated Ca²⁺ currents were similar in both groups (18.3 ± 2.3 pC vs. 20.2 ± 4.2 pC and 118 ± 18 pA vs. 112 ± 16 pA in mutated and control cells, respectively). This was further confirmed by measurements of cytosolic Ca²⁺, revealing no differences between mutated and control cells and indicating that Ca²⁺ handling played no role in the mutated islet phenotype. Glucose-induced (7.5 ± 2.7 vs. 10.4 ± 3.6, *P* = 0.1) or KCl-induced (9.2 ± 2.9 vs. 10.4 ± 2.0, NS) opening of voltage-dependent Ca²⁺ channels and resulting changes in cytosolic free Ca²⁺ concentration also did not differ between mutated and control islets (Supplemental Figure 3, A–C).

Third, we quantified membrane-docked granules using total internal reflection fluorescence (TIRF) microscopy in islets expressing the dense-core granule marker NPY-venus. TIRF allows the observation of single fluorescence-tagged vesicles at the cell membrane that undergo exocytosis and release their fluorescent cargo. The density of granules near the β cell plasma membrane did not differ between unstimulated mutated and control islets (0.99 ± 0.08 g/μm²; n = 24 cells vs. 0.92 ± 0.05 g/μm²; n = 56 cells, not shown), in agreement with a similar first-phase insulin secretion in both genotypes.

To analyze whether the mixed background of the experimental mice might be responsible for the increased insulin secretion observed in Pdx1Cre;Pparb/dfl/fl islets, we assessed GSIS in islets isolated from C57BL/6J mice, which were infected with adenovirus expressing shRNA against PPARβ/δ (Ad-shRNA-PPARβ/δ) and control shRNA (Ad-shRNA-control). Scale bar: 50 μm. DAPI in blue. Quantification of phalloidin fluorescence intensity in control and KO islets (C) and in control islets infected with Ad-shRNA-control and Ad-shRNA-PPARβ/δ viruses (D). Normalization was according to Glut2-expressing cells and number of nuclei in C and D, respectively (*n* = 3). **P < 0.01; ***P < 10⁻⁸. (E) Effect of 10 mM latrunculin (Lat) on GSIS in control and KO islets: (*n* = 3). ***P < 10⁻⁴; **P < 0.01.
Pdx1Cre; Pparβ/δ floxed islets via an adenovirus expression vector decreased insulin secretion in islets incubated at 16.7 mM glucose (Supplemental Figure 3E).

Collectively, these data demonstrate that Pparβ/δ deletion in the pancreas accelerated granule exocytosis and second-phase insulin secretion, while the initial size of the RRP remained unaffected. Importantly, these effects were independent of the mouse strains.

Global gene expression profile of PPARβ/δ-deficient islets. To examine the possible causes of disturbance in the complex second-phase insulin secretion, we used Affymetrix microarrays to compare mRNA expression profiles of islets isolated from 8-week-old Pdx1Cre; Pparβ/δ floxed (control) and Pdx1Cre; Pparβ/δfloxed (KO) mice. (B) Effect of 2.8 and 16.7 mM of glucose and 300 nM of PMA on PKD1 phosphorylation at serines 916 and 748 in islets from control and KO mice. (C) Representative pictures of immunostaining of giantin (red) in control and KO islets. Scale bar: 20 μm. (D) Quantification of giantin-stained areas normalized by the number of nuclei (DAPI) (n = 10–15 islets from 3 mice). ***P < 10−4. (E) PKD1 protein levels in control and KO islets infected with adenovirus expressing shRNA against PKD1 (Ad-shRNA-PKD1) and control shRNA (Ad-shRNA-control). (F) Representative photographs of control and KO islets infected with Ad-shRNA-control and Ad-shRNA-PKD1 adenovirus. Yellow arrow shows the tubular protrusions in Golgi apparatus. Yellow arrowhead shows normal nucleus-adjacent Golgi apparatus. Scale bar: 20 μm. (G) Quantification of giantin stained areas normalized by the number of nuclei (DAPI) in control (C) and KO islets infected with Ad-shRNA-control and Ad-shRNA-PKD viruses (n = 10–15 islets from 3 mice). ***P < 10−4.

Figure 7
Altered Golgi organization in PPARβ/δ-deficient islets. (A) PI4KIIIβ, PKD1 protein, pS748-PKD1, and pS916-PKD1 levels in islets isolated from Pparβ/δ floxed (control, C) and Pdx1Cre; Pparβ/δ floxed (KO) mice. (B) Effect of 2.8 and 16.7 mM of glucose and 300 nM of PMA on PKD1 phosphorylation at serines 916 and 748 in islets from control and KO mice. (C) Representative pictures of immunostaining of giantin (red) in control and KO islets. Scale bar: 20 μm. (D) Quantification of giantin-stained areas normalized by the number of nuclei (DAPI) (n = 10–15 islets from 3 mice). ***P < 10−4. (E) PKD1 protein levels in control and KO islets infected with adenovirus expressing shRNA against PKD1 (Ad-shRNA-PKD1) and control shRNA (Ad-shRNA-control). (F) Representative photographs of control and KO islets infected with Ad-shRNA-control and Ad-shRNA-PKD1 adenovirus. Yellow arrow shows the tubular protrusions in Golgi apparatus. Yellow arrowhead shows normal nucleus-adjacent Golgi apparatus. Scale bar: 20 μm. (G) Quantification of giantin stained areas normalized by the number of nuclei (DAPI) in control (C) and KO islets infected with Ad-shRNA-control and Ad-shRNA-PKD viruses (n = 10–15 islets from 3 mice). ***P < 10−4.
and the term cytoskeleton was enriched among downregulated genes. These findings may reflect a role of PPARβ/δ in cell chemotaxis, polarization, and migration, as observed in keratinocytes (27). Finally, genes related with vesicles and the Golgi system were enriched among upregulated genes in Pdx1Cre;Pparb/dββ islets, in agreement with enhanced insulin secretion by the mutated islets.

In order to validate the cytoskeleton and fatty acid gene expression profiles, quantitative PCR (qPCR) was performed on RNA samples from islets isolated from 8-week-old mice (Figure 5C). As observed in the microarray analysis, PPARβ/δ-deficient islets presented alterations in the expression of genes coding for the guanine nucleotide exchange factor–activated (GEF-activated) small GTPases implicated in the regulation of the cytoskeleton (Arfgef2, Rasgrp1, and Klar), the actin-polymerization regulators (Gin and Retn), and the granule-trafficking regulators (Rph3a and Rab3c). Also interestingly, markers for secretion (Chgb) and oxidative stress (Gpx3 and Mel) were increased in Pparβ/d-null islets. Similarly, fatty acid regulators in the Golgi membrane (Chpt1, Pld1, and Prkd1) also had an altered expression in PPARβ/δ-deficient islets.

We speculated that apo-PPARβ/δ mediates the unveiled broad repression activity on insulin secretion, which is similar to its inhibitory activity in early development during gastrulation (Nicolas Rotman and Walter Wahl, unpublished observations). In support of this hypothesis, treatment of islets with a PPARβ/δ ligand during GSIS increased secretion to levels observed in Pparβ/d-null islets (Figure 5D), which is consistent with an agonist-dependent alleviation of repression. The molecular mechanism of this effect remains to be investigated.

Based on the gene expression results and the interesting observations in insulin secretion, we then concentrated our study on the β cell insulin secretion pathway.

PPARβ/δ has an impact on the insulin secretory pathway at multiple sites. The enhanced second phase of secretion in the absence of PPARβ/δ suggests a higher flux of secretory granules to the plasma membrane for insulin release, which might be caused by an increased rate of granule fission at the trans-Golgi network (TGN), an unimpeded transport of insulin granules through the cortical actin meshwork, and a more efficient insulin exocytosis at the plasma membrane.

In fact, the gene expression–profiling data presented above provided support for important deregulations of genes with functions in granule biosynthesis, vesicle trafficking, and exocytosis. For instance, the most deregulated gene after Pparβ/d deletion encodes rabphilin (Rph3a) (Figure 5C), a Rab 3-GTP binding protein concentrated on secretory vesicles of neurons and endocrine cells (43). Similarly, Rab3c, also encoding a GTP-binding protein that colocalizes with secretory vesicles and plays a role in exocytosis (44), was upregulated. Furthermore, enhanced expression of the RAS guanyl releasing protein 1 (Rasgrp1) gene, which mediates the effects of acetylcholine through calcium and DAG binding, may also increase insulin secretion (45).

Polymerized cortical actin is known to form a barrier for insulin granule movement toward the plasma membrane, thereby limiting exocytosis (46, 47). Because GO analysis revealed that the expression of several cytoskeleton-related genes was decreased (Figure 5B), we analyzed the distribution of F-actin in β cells lacking PPARβ/δ. Quantification of the F-actin by phalloidin (Figure 5C) revealed that, compared with control islets (Figure 5A), Rab3c and Rad3c express 2.5- and 1.6-fold more PKD1 and PI4KIIIβ, respectively (Figure 5D). Furthermore, phosphorylation of PKD1 at the catalytic (serine-748) and autophosphorylation (serine-916) residues, known to result in a higher PKD1 activity, was increased not only in non-glucose-stimulated Pparβ/d-null islets (Figure 7A), but also in 30-minute glucose-stimulated islets (Figure 7B). As overactivation of PKD1 alters Golgi membrane structure (17), we monitored the localization of the Golgi marker giantin and the TGN marker TGN38 by immunofluorescence microscopy in isolated β cells (Figure 7, F and G). While in control islets, Pparβ/d null cells showed an extended and diffuse TGN38 staining pattern (48). To determine whether reduced F-actin in PPARβ/δ mutant islets promotes insulin secretion, we experimentally broke the cortical F-actin barrier. We assessed the effect of latrunculin, an F-actin–depolymerizing drug, on GSIS. Latrunculin increased 16.7 mM GSIS 11-fold in control islets, but only 4-fold in Pdx1Cre;Pparb/dββ islets (Figure 6E). This result is compatible with a higher level of F-actin and thus a stronger effect of latrunculin in PPARβ/δ WT compared with Pdx1Cre;Pparb/dββ islets. Taken together, these data implicate PPARβ/δ in an F-actin filament network that decreases GSIS in WT pancreatic β cells. We conclude that reduced actin filament polymerization in mutated cells is likely to increase second-phase insulin secretion.

Finally, the transcriptomic analysis indicated upregulation of several Golgi genes (Figure 5B), including Prkd1. Recent work revealed a key role of the Golgi-associated kinase PKD1 (also called PKCu) in TGN structure maintenance, export granule fission, and insulin secretion (17). Furthermore, PKD1 effects are mediated, at least in part, by phosphorylation of PI4KIIIβ (49, 50). These results prompted us to assess the impact of Pparβ/d deletion on the Golgi apparatus. We measured the protein level of PI4KIIIβ as well as the level and phosphorylated state of PKD1 in islets isolated from 8-week-old mice. Compared with control islets, PPARβ/δ-deficient islets expressed 2.5- and 1.6-fold more PKD1 and PI4KIIIβ, respectively (Figure 7A). Furthermore, phosphorylation of PKD1 at the catalytic (serine-748) and autophosphorylation (serine-916) residues, known to result in a higher PKD1 activity, was increased not only in non-glucose-stimulated Pparβ/d-null islets (Figure 7A), but also in 30-minute glucose-stimulated islets (Figure 7B). As overactivation of PKD1 alters Golgi membrane structure (17), we monitored the localization of the Golgi marker giantin and the TGN marker TGN38 by immunofluorescence microscopy in isolated β cells. In the absence of PPARβ/δ, both markers showed an extended and more diffused distribution compared with the expected compact nuclear proximal structures in the WT cells (Figure 7, C and D, and Supplemental Figure 5A). Expanded giantin distribution was not due to increased levels of the protein (Supplemental Figure 5D). To determine whether the PKD1 signaling pathway was indeed involved in this Golgi membrane alteration, Pparβ/d null and control islets were infected with adenovirus expressing siRNA against Prkd1. A decrease of the PKD1 level (Figure 7E) reversed the giantin distribution observed in PPARβ/δ-deficient β cells (Figure 7, F and G), while in control islets, it generated tubular protrusions on the Golgi apparatus, as reported previously (Figure 7F) (17). In addition, treatment with a PKD1 inhibitor (Go6976) reversed giantin distribution in PPARβ/δ-deficient β cells, which became similar to that in control islets (Supplemental Figure 5, B and C). These observations suggest that PPARβ/δ participates in mechanisms regulating membrane fission at the TGN through the regulation of PKD1 activity.

Collectively, the data gained from the analysis of the impact of PPARβ/δ on the insulin secretion pathway revealed what we believe are novel functions of this receptor. PPARβ/δ, most likely in its apo
form, plays an essential role in limiting β cell secretion by acting in a coordinated manner on at least 3 different steps of GSIS: the production of insulin granules from the TGN, the control of cortical actin as a barrier for granules toward the plasma membrane, and the production of regulators of insulin granule exocytosis.

**Discussion**

This study identifies a previously unrecognized role of PPARβ/δ in insulin secretion associated with a broad repressive effect on islet β cell gene expression. Removal of this inhibitory action by deletion of Pparb/d in pancreas led to a higher number of islets and enhanced insulin release during the second phase of GSIS. We propose that the unexpected consequent morphological disturbances of the β cell, namely actin cytoskeleton disorganization and altered Golgi apparatus, participated in this enhanced insulin release. Changes in the plasma levels of insulin and glucose in mice with Pparb/d-null pancreatic cells became apparent after weaning, when the pancreas normally reaches its adult size and has acquired its definitive morphology and functions.

Several observations reported herein support the notion that the enhanced insulin secretion reported in these mutant mice is due to the deletion of the Pparb/d gene in β cells rather than in non-β cells, even though similar expression levels of PPARβ/δ in α and β cells were noted in WT mice. Thus, increased GSIS and cytoskeleton disorganization in cultured C57BL/6j islets in which PPARβ/δ was knocked down make a role of acini in the PPARβ/δ-controlled regulation of these processes unlikely. Furthermore, capacitance measurements performed in single cells from dissociated islets, which were electrophysiologically identified as β cells, revealed enhanced exocytosis in PPARβ/δ-deficient β cells, excluding a direct role of α cells in the accelerated exocytosis. PPARβ/δ regulates pancreatic β cell mass in neonatal stages. Pancreatic deletion of Pparb/d did not alter the development of islets at the embryonic, but at the neonatal stage. Pancreatic insulin content and DNA expression of endocrine markers (Supplemental Table 1) were normal in PPARβ/δ-deficient pancreas at P3. In contrast, these mice presented an increase of RNA expression of endocrine markers and β cell proliferation at P14, suggesting an important role of PPARβ/δ in β cell mass regulation in neonatal stages, which correlates with the enhanced expression of this nuclear receptor in pancreas at P14. Interestingly, at 8 weeks of age, the β cell mass remained higher in the mutated pancreata, but β cell proliferation returned to normal. Hence, Pparb/d deletion in pancreas may alter the neonatal remodeling of β cell mass, which normally occurs from P9 to P31 in rat (51). This remodeling period is likely to account for the enhanced β cell mass at 8 weeks of age in the mutated mice. Decreased p27Kip1 mRNA expression in PPARβ/δ-deficient islets (fold change –1.5; P < 0.003) may underlay the accelerated proliferation at neonatal stages in the mutant mice. Interestingly, overexpression of p27Kip1 reduces pancreatic β cell proliferation only when applied during neonatal and postnatal development (52), suggesting a special gene expression program regulating postnatal β cell mass. How PPARβ/δ is implicated in this program remains to be determined.

The absence of PPARβ/δ affects second-phase insulin release. Altered β cell mass does not necessarily correlate with disturbances in plasma insulin levels (40). Hence, we hypothesized that insulin secretion changes may contribute to the hyperinsulinemia in 8-week-old PPARβ/δ-deficient mice. We found both enhanced second-phase insulin secretion from perfused islets and an increased rate of sustained exocytosis in Pparb/d-null β cells as assessed by capacitance measurements. Changes in Ca2+ handling do not explain enhanced GSIS in Pparb/d-null islets because evoked Ca2+ currents and measured intracellular [Ca2+]i were similar in PPARβ/δ mutant and control islets. Also, TIRF microscopy analysis showed similar numbers of docked granules in control and PPARβ/δ-deficient islets, indicating that the formation of the RRP of granules proceeds normally. In contrast, this suggested an increased efficiency of recruitment and exocytosis of secretory granules coming from internal stores and/or newly formed at the TGN. Indeed, microarray analysis provided support for multiple other mechanisms augmenting insulin secretion in the mutated islets.

First, the exocytic step may be increased because of the dramatically higher expression of raphphilin 3a (Figure 5C), as has been shown in β cells with forced overexpression of this protein (43, 53). There was also a 1.4-fold decrease of neurexin 1α (P < 0.004) in PPARβ/δ-deficient islets. This protein functionally interacts with granuphilin in the docking of secretory granules. In addition, mice null for neurexin 1α present an increased second-phase insulin secretion (54).

Second, second-phase insulin secretion requires mobilization of RP granules during prolonged glucose stimulation. These granules must cross the F-actin network beneath the cell surface to reach their release site at the cell membrane. Granule mobilization for RRP refilling is tightly associated with F-actin remodeling, which involves actin filament disassembly (55). Here, we observed actin cytoskeleton disorganization in Pparb/d-null islets with reduced basal amounts of F-actin in the absence of glucose stimulation. This drastic alteration in the F-actin network already at basal levels (before glucose stimulation) suggests that other factors to be identified have a dominant impact on F-actin in Pparb/d-null β cells. We propose that this defect in cytoskeleton disorganization facilitates second-phase insulin secretion. In agreement with this conclusion, we have shown that an actin depolymerization agent (latrunculin) potentiates GSIS in WT islet much more than in PPARβ/δ-deficient islets, which is consistent with strongly reduced F-actin in the latter.

Third, Pparb/d-null β cells also display an alteration of the Golgi network, and our study showed that this was caused by an overactivation of the Golgi-associated PKD1. PKD1 regulates membrane fission at the TGN, promoting formation of transport vesicles and priming them for efficient routing to the cell membrane (56). P4KIIIβ is an essential target of PKD1 action in this vesicular trafficking (50). Pparb/d-null β cells show an increase in expression of both proteins and phosphorylation of PKD1. This could account for part of the augmented secretory activity of these islets, as shown in mice lacking the MAPK p38β, which exhibit an increased insulin secretion phenotype similar to that described here (17). This defect was demonstrated to result from lack of phosphorylation of PKD1 on inhibitory serine residues, leading to overactivity of this kinase, deregulated Golgi structure, and increased insulin secretion. Also, DAG is required for activation of PKD1 to promote vesicular fission from TGN (49). Enzymes implicated in the accumulation of DAG on TGN, as sphingomyelin synthase 1 and DAG cholinephosphotransferase 1, showed 1.5-fold (P < 0.001), and 2.2-fold (P < 10^-6) increases of their mRNA in PPARβ/δ-deficient islets, respectively. Therefore, we propose that increased PKD1 levels and activity in mice lacking PPARβ/δ affect the Golgi/TGN without influencing insulin production, which is in agreement with unmodified insulin content in the mutated β cells.
Conclusion. Our work revealed what we believe are novel and thus-far-recognized roles for PPARβ/δ in pancreatic β cell functions. We used a pancreas-specific deletion of Pparb/d to confirm that the changes we observed are not attributable to indirect effects that would arise from a whole-organism Pparb/d deletion. A first insight into PPARβ/δ functions came from gene expression profiling, which unveiled an unexpectedly broad gene expression-regressive function of this receptor isotype in β cells, most likely in its apo form. The second insight was that the systemic impact of depleting pancreatic PPARβ/δ, i.e., hyperinsulinemia and lower glycemia, results from alterations in basic cellular functions, such as Golgi functions and actin cytoskeleton-dependent routing of granules to the cell periphery. In fact, this study identified PPARβ/δ as a master regulator of functions associated with each step of insulin secretion, i.e., granule biosynthesis, vesicle trafficking, and exocytosis. Targeting PPARβ/δ with tissue-specific modulators might offer a novel approach to stimulating β cell survival and insulin release under conditions of sustained glucose stimulation as encountered in type 2 diabetes.

Methods

Mouse models. Pparb/dfl/fl mice (35) having exon 4 (which encodes the N-terminal zinc finger of the DNA-binding domain) of the Pparb/d gene flanked by loxP sites were mated with mice carrying the Cre transgene under the control of the Pdx1 promoter, which we refer to as Pdx1Cre (38). The Pdx1Cre ‘Pparb/dfl/fl’ progeny males were bred with Pparb/dfl/fl females to obtain Pdx1Cre ‘Pparb/dfl/fl’ mice. To maintain this genotype, we mated the Pdx1Cre ‘Pparb/dfl/fl’ males with Pparb/dfl/fl females. Some Pparb/dfl/fl mice were bred with Pdx1Cre/R26R to obtain Pdx1Cre ‘Pparb/dfl/fl’;R26R. Pdx1Cre mice were mated with Pdx1Cre ‘ mice (WT) to maintain the Pdx1Cre and WT control mice. The Pdx1Cre and R26R transgenes (57) were maintained in hemizygote conditions. All the mice were bred on a C57BL/6 background.

Metabolic studies and endocrine hormone content in pancreas and islets. Blood collection from the tail vein was performed in fed mice between 8:00 and 10:00 am. Blood collection from P3 mice was carried out by decapitation. Insulin determination was performed with 10 μl of plasma or 10 μl of 1/5,000 pancreatic acid–ethanol extracts by using an ultrasensitive mouse insulin ELISA. For glucagon determination, we used 50 μl of plasma or 50 μl of 1/1,000 pancreatic acid–ethanol extracts by using RIA (Linco). Glycemia was measured with the Accu-Chek Aviva System (Roche Diagnostics). The GTT was performed in animals fasted for 12 hours by i.p. injection of glucose (2 mg/g), and at the indicated time, the glycemia was assayed from tail blood samples. For the insulin secretion experiments, mice fasted 12 hours were i.p. injected with glucose (3 mg/g), as described for GTT experiments. Regarding ITT, chow-diet–fed animals were i.p. injected with insulin (0.75 U/g), and glucose determination was done at the indicated time. Insulin and glucagon content were determined from 30 sonicated islets in lysate buffer (TRIS buffer, pH 7.5, and 1% Triton X-100) by ELISA (Merckodia) and RIA (Linco), respectively. In islets, insulin content was normalized by DNA quantification with Hoechst, whereas glucagon content was normalized by total protein amount with BCA.

Immunohistochemistry in pancreas and islets. Pancreata from embryos (E15.5 and E17.5) or adult mice (2 and 8 weeks of age) and ECM-plated islets were washed with PBS and fixed immediately in 4% paraformaldehyde at 4°C overnight and 10 minutes, respectively. X-gal staining and immunohistochemistry procedures are described in Supplemental Methods. The primary antibodies were used at the following dilutions: guinea pig anti-swine insulin (Dako), 1/400; rat anti-e-cadherin (Abcam), 1/1600; rabbit anti-Glut2 (gift of Bernard Thorens, Center for Integrative Genomics, University of Lausanne, Switzerland), 1/1000; rabbit anti-giantin (Covance Inc.), 1/1000; sheep anti-TGN38 (AbD Serotec), 1/500; rabbit anti-Neurog3 (Santa Cruz Biotechnology Inc.), 1/1000; and rabbit anti-PPARβ/δ (Thermo Scientific Inc.), 1/200.

Determination of β cell mass and proliferation. Pancreata from 3 Pdx1Cre ‘Pparb/dfl/fl’ and 3 Pparb/dfl/fl 8-week-old mice were processed to determine the islet β cell mass by using the following equation: β cell mass = pancreas weight (mg) × relative insulin surface (total islet area μm²/total pancreas area μm²) × 100. For details see Supplemental Methods. Pancreata from 2- and 8-week-old mice, previously injected with BrdU, were sectioned and stained against anti-BrdU (Roche Applied Science) and anti-insulin. The sections were analyzed by confocal microscopy (Zeiss) and BrdU–insulin-expressing cells were counted with ImageJ.

Fluorescence quantification. For staining of F- and G-actin, giantin, Glut2, and TGN38, we plated 10–15 islets for mice (n = 3) on ECM (Novamed) in RPMI medium for 2 days in the case of KO mice or 3 days for adenovirus-infected islets. For F-actin and G-actin determination, we performed phalloidin–rhodamine (Invitrogen), and DNAse I Alexa Fluor 488 conjugate (Invitrogen) staining, respectively. Giantin staining was performed in adenovirus-infected or noninfected islets that were incubated with or without 1 μM of Go6976 (Calbiochem) for 12 hours in RPMI 1640 medium supplemented with 5 mM of glucose. Stained islets were imaged by confocal microscopy (Zeiss); phalloidin fluorescence intensity and giantin-stained area were quantified by ImageJ software and normalized by Glut2–expressing cells and DAPI-stained nuclei, respectively.

Pancreatic islets isolation and glucose insulin-stimulated secretion. Islet isolation was accomplished from 8-week-old mice by 100 mg of collagenase digestion (211 U/mg) followed by purification through a Histopaque gradient. Islet perfusion was carried out by placing 30 islets into a perfusion chamber (Millipore) as described previously (58). To increase the glucose secretion, we supplemented the Krebs-Ringer bicarbonate buffer (KRBB) with 50 μM of 3-isobutyl-1-methylxanthine (IBMX). We determined the insulin concentration by RIA from each fraction collected. The insulin secretion was normalized with the insulin content that remained in the islets after perfusion. GSIS was performed by preincubating 10 adenovirus-infected or noninfected islets in KRBB, pH 7.4, supplemented with 2.8 mM of glucose, with or without 10 mM ofatrulcinulin (Calbiochem) for 1 hour at 37°C, in triplicate for each mouse (set of 10 islets).

Then, we incubated the islets in KRBB with 2.8 or 16.7 mM of glucose, with or without latrunculin, for 1 hour at 37°C. For PPARβ/δ agonist treatment, the islets were starved for 16 hours in RPMI without PBS before incubation with or without 100 nM of GW501516 for 24 hours at 37°C. Then the islets were incubated in glucose as above. Finally, we determined the insulin secreted in the buffer by using an ultrasensitive mouse insulin ELISA; the insulin secretion was normalized by total insulin content from islets (acid-ethanol preparation).

Adenovirus infection of pancreatic islets. Islets were isolated as previously described and infected with recombinant adenoviruses at a multiplicity of infection of 200, 48 hours before utilization. After isolation, islets were gently dissociated using HBSS containing 5 mM glucose 1 mM EGTA at 37°C for 3 minutes before being put in contact with recombinant adenoviruses: 200 islets per well in a 12-well plate (nunc NC-150200) for 3 hours at 37°C. To overexpress PPARβ/δ, we used recombinant adenoviruses expressing GFP alone or human PPARβ/δ-GFP (Vector Lab), both under CMV promoter control. To knock down Pparb/d and Prdk1, we used recombinant adenoviruses coexpressing GFP with a shRNA mouse sequences 5′-CAGATCTACAAGGCTCAT-3′ and 5′-CAGGAGAGATGAGATCAT-3′ against Pparb/d and Prdk1, respectively.


More information at www.jci.org/articles/view/42127
RNA preparation from pancreatic α and β cells sorted by FACS. Pancreata were injected with 5 ml collagenase V (0.5 mg/ml) in Ca²⁺- and Mg²⁺-free HBSS, dissected from the surrounding tissue, and transported on ice. Following an 11-minute digestion at 37°C, the pancreas was disrupted by vigorous shaking, and the islets were transferred by manual picking into HBSS containing 10 mM glucose and 0.1% BSA. Islets were disrupted by trituration in 0.1X trypsin/EDTA, and 10% FCS was then added. Cells were separated immediately using a MoFlo Beckman Coulter Cytomation Sorter (488 nm excitation). Single cells were selected by their forward scatter and pulse width, α cells were selected on the basis of their relative Venus fluorescence at 530 and 580 nm, and β cells were selected by their high side scatter, absence of Venus fluorescence, and high background autofluorescence at 530 nm (59). From 10, 000 to 30,000 cells were collected into lysis buffer (Ambion), and the total was isolated using a microscale RNA isolation kit (Ambion).

**Capacitance and intracellular Ca²⁺ measurements.** Measurements of exocytosis were performed in the whole cell configuration using an EPC-9 amplifier controlled by Pulse software (both HEKA Elektronik). Intracellular Ca²⁺ measurement was performed by fluorescence in islets incubated with FURA-RED AM (Invitrogen). For details, see the Supplemental Methods.

**TaqMan qRT-PCR and microarray.** Total RNA from islets or pancreata was extracted using guanidine thiocyanate (Sigma-Aldrich). For qRT-PCR, we used TaqMan probes (Applied Biosystems) and primers for SYBR-green detection. cDNA from human islets was obtained from M. Prentki’s laboratory (Montreal Diabetes Research Center and CRCHUM, and the Departments of Nutrition and Biochemistry, University of Montreal). For microarray analysis, we amplified 5 ng of total islet RNA (from each mouse) and labeled it using the GeneChip IVT labeling kit according to the protocol provided by the supplier. For details, see Supplemental Methods. Microarray data have been deposited (GEO GSE16048).

**Western blot analysis.** Approximately 300 islets were lysed in lysis buffer supplemented with protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors. A total of 10–20 μg of total protein extracts were resolved by SDS–10% polyacrylamide gels and electrotransferred onto a nitrocellulose membrane (Whatman). We developed the membrane using a chemiluminescence ECL system (Amersham), with anti-PHKLII (Millipore), anti-PKD1-1-spc 748 (Abcam), anti-Neurog3 (Santa Cruz Biotechnology Inc.), anti-giantin (Covance Inc.), anti-PKD1, anti-PKD1-1-spc 916, and anti-β-actin from Cell Signaling Technology. To assess the effect of glucose and PMA on PKD1 phosphorylation, we processed the islets in the same way as in the GSIIS experiment, but the incubation was for 30 minutes at 2.8 or 16.7 mM of glucose or 300 nM of PMA as positive control. 20 μg of total protein extracts were resolved in a gel, as mentioned above.

**Statistics.** The data are presented as mean ± SEM. Statistical analysis was performed using Student’s t test. In all the tests, we compared 2 groups and assumed a 2-tailed distribution and unequal variance. Values were considered significant at P < 0.05. The statistical tests for microarray analysis are described in Supplemental Methods.

**Study approval.** All experiments involving animals were reviewed and approved by the Veterinary Office of the Canton Vaud (SAC–EXAPINAM, Service de la Consommation et des Affaires Vétérinaires, Epalinges, Switzerland) in accordance with the Federal Swiss Veterinary Office Guidelines. The utilization of human islets was approved by the Comité d’Éthique de la Recherche sur les Sujets Humains, CRCHUM.

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